Nuclear Transport of U1 snRNP in Somatic Cells: Differences in Signal Requirement Compared with *Xenopus laevis* Oocytes

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Abstract. The signal requirement for the nuclear import of U1 RNA in somatic cells from different species was investigated by microinjection of both digoxygenin-labeled wild type and mutant U1 RNA molecules and in vitro reconstituted U1 snRNPs. U1 RNA was shown to be targeted to the nucleus by a temperature-dependent process that requires the prior assembly of RNPs from the common proteins and the microinjected RNA. Competition in the cell between immunoaffinity-purified U1 snRNPs and digoxygenin-labeled U1 snRNPs reconstituted in vitro showed that the transport is saturable and should therefore be a mediated process. The transport of a karyophilic protein under the same conditions was not affected, indicating the existence of a U snRNP-specific transport pathway in somatic cells, as already seen in the *Xenopus laevis* oocyte system. Surprisingly, the signal requirement for nuclear transport of U1 snRNP was found to differ between oocytes and somatic cells from mouse, monkey and *Xenopus*, in that the m_3GGpppG-cap is no longer an essential signaling component in somatic cells. However, as shown by investigation of the transport kinetics of m_3GpppG- and ApppG-capped U1 snRNPs, the m_3GpppG-cap accelerates the rate of U1 snRNP import significantly indicating that it has retained a signaling role for nuclear targeting of U1 snRNP in somatic cells. Moreover, our data strongly suggest that cell specific rather than species specific differences account for the differential m_3G-cap requirement in nuclear import of U1 snRNPs.

At the nuclear envelope, continual exchange of macromolecules between the nucleus and the cytoplasm takes place. For large components, such as proteins, RNAs, and RNA–protein complexes (RNPs),[1] this transport is signal mediated and saturable and hence a receptor-mediated process. (Feldherr et al., 1984; Goldfarb et al., 1986; Forbes, 1992). Up to now, the transport of proteins into the nucleus is the best understood of these processes (reviewed in Garcia-Bustos et al., 1991; Silver, 1991). It occurs in two separate steps: the initial binding of the protein to the nuclear pore complex, followed by an ATP-dependent translocation of the protein into the nucleus (Richardson et al., 1988; Newmeyer and Forbes, 1988).

The information contained in nuclear location signals (NLS) allows the selective interaction of karyophilic proteins with import receptors (reviewed in Garcia Bustos et al., 1991; Silver, 1991; Nigg et al., 1991; Forbes, 1992). Some nuclear proteins bear signals composed of a single short basic sequence resembling the NLS of the SV-40 large T-antigen (Lanford and Butel, 1984; Lanford et al., 1986; Kalderon et al., 1984) while others contain a more complex bipartite NLS first defined in nucleoplasmin (Robbins et al., 1991).

In addition to karyophilic proteins, RNA–protein complexes such as the spliceosomal U snRNPs U1, U2, U4, and U5 are a further major group of macromolecules which are selectively targeted to the nucleus.

The snRNAs U1-U5 are all transcribed by RNA polymerase II and share two structural motifs: the m_3GpppG-cap structure (m_3G-cap) containing the $m^{2,2,7}$-trimethylguanosine (Reddy and Busch, 1988) and a single-stranded uridylic acid-rich sequence referred to as the Sm-binding site (Branlant et al., 1982; Liautard et al., 1982). Two classes of proteins bind to the individual snRNAs. One group of common (Sm) proteins designated B, B′, D1, D2, D3, E, F, and G are present in all U snRNPs (Lührmann et al., 1990). They bind to the Sm site of the snRNA forming the Sm core domain which is morphologically similar among all spliceosomal U snRNPs (Kastner et al., 1990; Lührmann et al., 1990). The second class is comprised of specific proteins which bind only to one particular U snRNA species (Lührmann et al., 1990).

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1. *Abbreviations used in this paper*: m_3G-cap, m_3GpppG-cap; NLS, nuclear location signals; RNP, RNA-protein complexes.

The morphogenesis of the U snRNP particles requires the export of the nuclear encoded m^7G-capped U snRNA to the cytoplasm followed by the association of these RNAs with the Sm proteins and hypermethylation of the m^7G- to the m_3G-cap (DeRobertis, 1983; Mattaj and DeRobertis, 1985; Mattaj, 1988). The snRNP particle then returns to the nucleus. In *Xenopus laevis* oocytes the nuclear location signal of U1 snRNP is bipartite with the m_3G-cap as one essential signaling component (Fischer and Lührmann, 1990; Hamm and Mattaj, 1990). The second part of the signal is located within the Sm core domain but has not yet been precisely defined (Fischer et al., 1993). Competition studies revealed that U1 snRNP and karyophilic proteins do not compete for common transport factors and therefore follow different kinetic transport pathways (Michaud and Goldfarb, 1991, 1992; Fischer et al., 1993). Surprisingly, not all spliceosomal snRNAs have the same m_3G-cap requirement for nuclear transport in oocytes. Whereas U1 and U2 cannot enter the nucleus without an intact m_3G-cap, this structure has a much less pronounced influence on the transport of U4 and U5. The latter RNAs can enter the nucleus as ApppG-capped derivatives, although with reduced transport kinetics (Fischer et al., 1991). The differential requirement of the m_3G-cap for U1 and U5 transport is unlikely to be due to differences in the activity of the Sm core NLS of both snRNP types, since U1 and U5 snRNPs compete for the same transport receptor. Rather the structure of the RNAs appears to be important for the requirement for an m_3G-cap as an essential signaling component (Fischer et al., 1993; Jarmolowski and Mattaj, 1993).

In the *X. laevis* oocyte, the various snRNA molecules exhibit different signal requirements for nuclear transport. It may therefore be asked whether the requirements for transport for the individual snRNAs differ from one cell type to another. For karyophilic proteins, several cases have already been described where the NLS activity differs between cell types or stages of development (Slaviak et al., 1989; Standiford and Richter, 1992). For example, the adenovirus 5 E1A protein contains two NLSs, of which one is constitutively active while the other appears to be regulated during embryogenesis of *X. laevis* (Standiford and Richter, 1992).

Among snRNAs, it has been reported that in somatic cells U2 RNA may be transported to the nucleus in an m_3G-cap–independent manner after transfection into human 293 cells (Kleinschmidt and Pederson, 1990). However, this study left open both the possibility that the transfection procedure per se affected the transport process and the alternative interpretation that the accumulation in the nucleus of the RNA introduced was due to cell divisions that might have occurred in the course of their experiments.

In this report we have studied the nuclear transport of U1 RNA molecules labeled with digoxygenin and U1 snRNP particles reconstituted in vitro. Both were microinjected into living somatic cells from different species. It is shown that the transport occurs in a temperature-dependent and saturable fashion, for which the assembly of the common proteins onto the U1 RNA is an essential prerequisite. The transport of U1 snRNP requires different limiting transport factors than those required for the transport of karyophilic proteins. The signal required to target the U1 snRNP-particle in somatic cells is shown to consist of the Sm core domain and the m_3G-cap. However, in contrast to nuclear transport of U1 snRNP in the oocyte, the cap has no essential function, but accelerates the transport kinetics significantly.

Materials and Methods

In Vitro Transcription

In vitro transcription of U snRNA genes. The clones XeU1, XeU1ΔD, and pSmII were linearized with BamHI or in the case of pSmII with AvaI and used for in vitro generation of snRNAs. XeU1 and XeU1ΔD RNAs were generated by T7-, and pSmII RNAs by SP6 transcription, respectively. In a typical 100-μl transcription assay 10 μg of linearized DNA template was incubated in buffer containing 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 25 mM DTE, 0.1 μg/μl BSA, 10 mM of the indicated cap dinucleotide, 2 mM each of GTP, ATP, and CTP, 1.5 mM UTP and 0.5 mM digoxygenin-UTP, and 1 U/μl polymerase. Transcription was allowed to proceed for 90 min at 37°C and stopped hereafter by adding 1 U/μl RNAse free DNAse I and continuing the incubation for 15 min at 37°C. RNA was phenol-extracted and precipitated with 3 vol of ethanol. Transcription efficiency was analyzed by separation of 2.5 μl of the transcription assay in an 0.8% TAE agarose gel and visualizing the bands with 2 μg/ml ethidium bromide. The typical yield of one transcription reaction was between 5 and 30 μg RNA.

In Vitro Reconstitution of Digoxygenin-labeled U snRNPs

In vitro reconstitution of U snRNPs was carried out essentially as described in Sumpter et al. (1992) with the exception that digoxygenin-labeled instead of ^{32}P-labeled U sn RNAs were used. In brief, snRNP proteins were isolated by incubation of 10 mg immunoaffinity purified U snRNPs with 30 ml DEAE cellulose (DE53; Whatman Laboratory Products Inc., Clinton, NJ) in buffer containing 150 mM KOAc, 140 mM NaCl, 5 mM EDTA, and 0.5 mM DTE. After incubation for 15 min on ice and a further 15 min at 37°C the DEAE-cellulose was pelleted by centrifugation. The supernatant contained the snRNP proteins. The solution was dialyzed against buffer containing 20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, and 0.5 mM DTE. The native U snRNP proteins were concentrated using a Centriprep concentrator3 (Amicon Corp., Arlington Heights, IL) in the first concentration step and Centricon C3 (Amicon Corp.) in the second to obtain snRNP proteins in concentrations up to 5 mg/ml. The yield was typically 1 mg native proteins from 10 mg U snRNP-particles. In vitro reconstitution was carried out by incubation of 0.5 μg (~9 pmol) U snRNA with 5 μg snRNP proteins in 3 μl reconstitution buffer for 30 min at 30°C and 15 min at 37°C. The in vitro reconstituted U snRNP particles were microinjected without further purification.

Cells and Microinjection

Vero (African green monkey kidney) and 3T3 cells were grown in Dulbecco's modified Eagle medium (GIBCO BRL, Bethesda, MD) supplemented with 10% fetal calf serum, 5% CO_2 at 37°C. *Xenopus* A6 cells were grown in L19 medium (GIBCO BRL) supplemented with 10% fetal calf serum, 5% CO_2 at 26°C. For microinjection experiments Vero, A6, and 3T3 cells were plated on glass coverslips in 35 mm dishes with 3 ml medium and grown to 80% confluence. Microinjection was carried out as described by Graessmann et al. (1980) using an Eppendorf micromanipulator 5170 and microinjector 5242 and Eppendorf Femtotips.

The amount of RNA injected in a typical experiment varied between 1-5 × 10^5 molecules per cell. The injection volume was ~200 fl per cell.

Immunofluorescence

Cells were prepared for immunofluorescence by flooding the coverslips with ice-cold 3% paraformaldehyde dissolved in PBS (pH 7.4) for 15 min and permeabilized in 0.2% Triton X-100 in PBS, pH 7.4, for 10 min. Cells were blocked in 10% fetal calf serum in PBS, pH 7.4, for 1 h at room temperature and incubated with an anti-digoxygenin antibody (5 mg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h. The cells were incubated for 30 min with an FITC-labeled anti-mouse IgG (Amersham Corp.).

Stained cells were mounted in mounting medium (Fluoroprep; BioMerieux, France) and photographs were taken on a Leitz Axioplan micro-

scope. Cells injected with fluorescent-labeled BSA–NLS conjugate were fixed and analyzed directly.

Fluorescence intensities in the nuclear and cytoplasmic fractions of fixed microinjected Vero cells were determined using a digitalized computer system. Nucleo-cytoplasmic ratios were calculated by dividing the fluorescence intensity of the nuclear and cytoplasmic compartment.

Results

Nuclear Import of U1 snRNAs to the Nucleus Is a Temperature-dependent Process Which Requires Binding of the Common Proteins to the RNA

We recently reported that the transport of RNA in somatic cells could be investigated by immunofluorescent microscopy after microinjection of in vitro transcribed RNA molecules labeled with digoxygenin (van Zee et al., 1993). This procedure has been implemented in the present study to analyze the signal requirements of U snRNA transport in somatic cells of diverse species.

Using Vero (African green monkey kidney) cells we initially analyzed the extent of nuclear import of m_3G-capped digoxygenin-U1 RNA 3 h after its microinjection into the cytoplasm. As shown in Fig. 1 *a*, m_3G-capped U1 RNA was transported efficiently to the nucleus. The U1 RNA detected in the nucleus was localized primarily in the nucleoplasm and excluded from the nucleoli. In cells which had received smaller amounts of digoxygenin-U1 RNA, we observed a nuclear staining pattern characteristic of endogenous U1 snRNAs (Carmo-Fonseca et al., 1991*a*,*b*). As a control, we carried out the same experiment at 4°C instead of 37°C. Under these conditions, the microinjected RNA was localized almost exclusively in the cytoplasm which makes the possibility unlikely that diffusion is responsible for the observed transport (Fig. 1 *b*).

In *X. laevis* oocytes nuclear import of U1 RNA requires the prior assembly of the Sm core domain in the cytoplasm (Mattaj, 1988). To investigate the contribution of the Sm core domain to U1 RNA import in somatic cells, we examined the transport behavior of the U1 RNA mutant designated U1ΔD. The common Sm proteins fail to bind to U1ΔD due to a substitution in the Sm site (Mattaj and DeRobertis, 1985; Mattaj, 1986). As shown in Fig. 1, *c* and *d*, U1ΔD was almost completely excluded from the nucleus 3 h after injection into the cytoplasm, irrespective of the nature of the 5′-terminal cap structure, as neither ApppG- (*c*) nor m_3GpppG-capped transcripts (*d*) were transported to the nucleus. Further support for the hypothesis that binding of the Sm proteins plays a major role in nuclear targeting of U1 RNA was provided by the following experiment. Co-injection of the monoclonal anti-Sm antibody Y12—which reacts with the common proteins B, B′, and D (Lerner and Steitz, 1981)—with U1 RNA drastically inhibited the transport of U1 RNA (Fig 1 *e*). Antibody C383, which reacts with the ribosomal protein S1 but not with snRNPs, in contrast did not inhibit transport of U1 RNA (Fig. 1 *f*). The most likely explanation for this result is that the antibody Y12 either inhibits the assembly of the common proteins on the U1 RNA molecule or binds to the in situ assembled U1 snRNP particle directly and masks the signals required for import. In either case, this finding strongly indicates that the assembly of the Sm core domain in the cytoplasm is a necessary event in targeting the U1 snRNP particle to the nucleus in Vero cells.

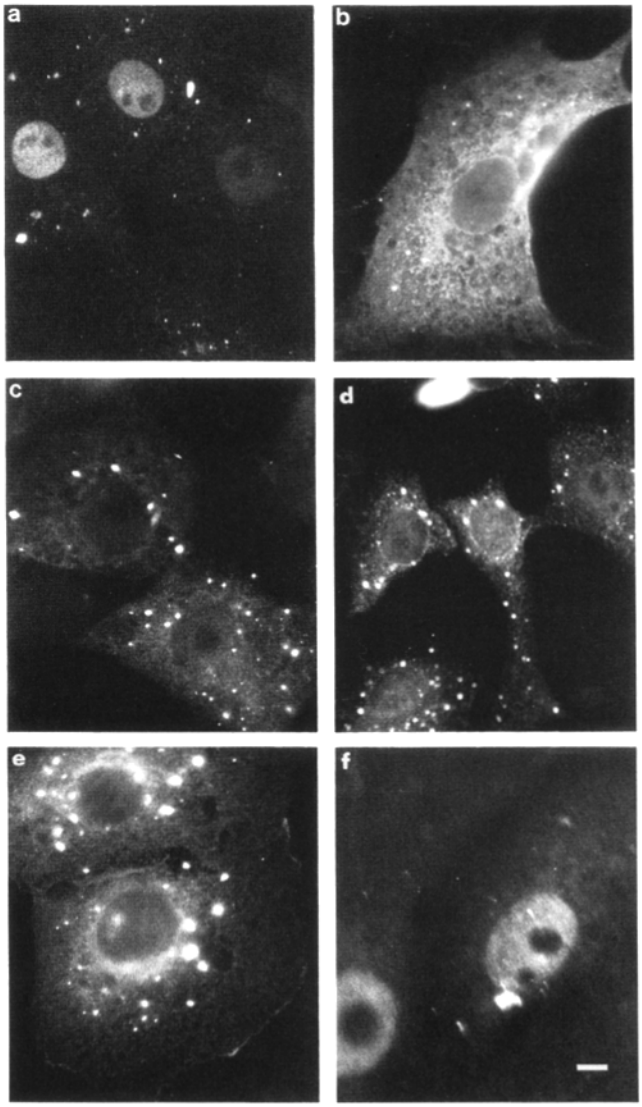

Figure 1. Digoxygenin-labeled U1 RNA transport to the nucleus of Vero cells depends on the assembly of the Sm proteins. Vero cells were microinjected with digoxygenin-labeled m_3GpppG-capped U1 RNA (*a* and *b*), or a mutant lacking the Sm site (U1ΔD) and carrying either an ApppG- (*c*), or an m_3GpppG-cap (*d*). In Fig. 1, *e* and *f*, digoxygenin-labeled m_3GpppG-capped U1 RNA was coinjected with the monoclonal antibody Y12, which recognizes the common proteins B, B′, and D1 or with the antibody C383 directed against the ribosomal protein S1 respectively. Every cell received an injection of ~1–5 × 10^5 RNA molecules. Cells were incubated for 3 h at 37°C (*a*, *c–e*) or 4°C (*b*) and then fixed and stained with anti-digoxygenin-antibody and FITC conjugated anti-mouse antibody. Bar, 10 μm.

We have recently shown that U1 snRNPs reconstituted in vitro from U1 RNA and native snRNP proteins were bona fide substrates for the nuclear transport apparatus of *X. laevis* oocytes (Fischer et al., 1993). The in vitro reconstituted U1 snRNP particles used for these studies contained all the Sm proteins and substoichiometric amounts of U1-specific proteins. For nuclear transport studies these parti-

cles offer a major advantage, in that the structural requirements of U snRNPs for transport can be determined in the absence of the in situ assembly requirements. Thus, we improved the in vitro reconstitution conditions for U1 snRNP to obtain digoxygenin-labeled U1 snRNPs sufficiently concentrated for microinjection studies in somatic cells. When in vitro reconstituted U1 snRNPs containing an m^7G-capped digoxygenin-labeled U1 RNA were injected into the cytoplasm of Vero cells, they were targeted efficiently to the nucleus within 3 h, demonstrating that they were efficient substrates for the nuclear transport apparatus (Fig. 2 *a*).

Biochemical studies in the oocyte system have previously shown that the reconstituted U1 snRNPs were transported as such and that disassembly/reassembly processes were unlikely to occur under these conditions (Fischer et al., 1993). While similar biochemical studies were not feasible with somatic cells, the following experimental evidence suggests that the in vitro reconstituted U1 snRNPs remained intact after injection into somatic cells. When we compared the kinetics of transport of U1 RNA microinjected either as naked RNA or as U1 snRNP reconstituted in vitro it became apparent that the reconstituted U1 snRNP particle was targeted more efficiently to the nucleus (Fig. 2 *B*). While the free U1 RNA needed at least 3 h for complete nuclear accumulation (Fig. 2, *f–h*), the U1 snRNP particle was already transported to a comparable extent after 1 h (Fig. 2, *c–e*). The most likely explanation for this observation is that the in vitro reconstituted U1 snRNP particle was transported as such to the nucleus. Further support for this notion that reconstituted snRNPs are transported as intact particles to the nucleus is presented below.

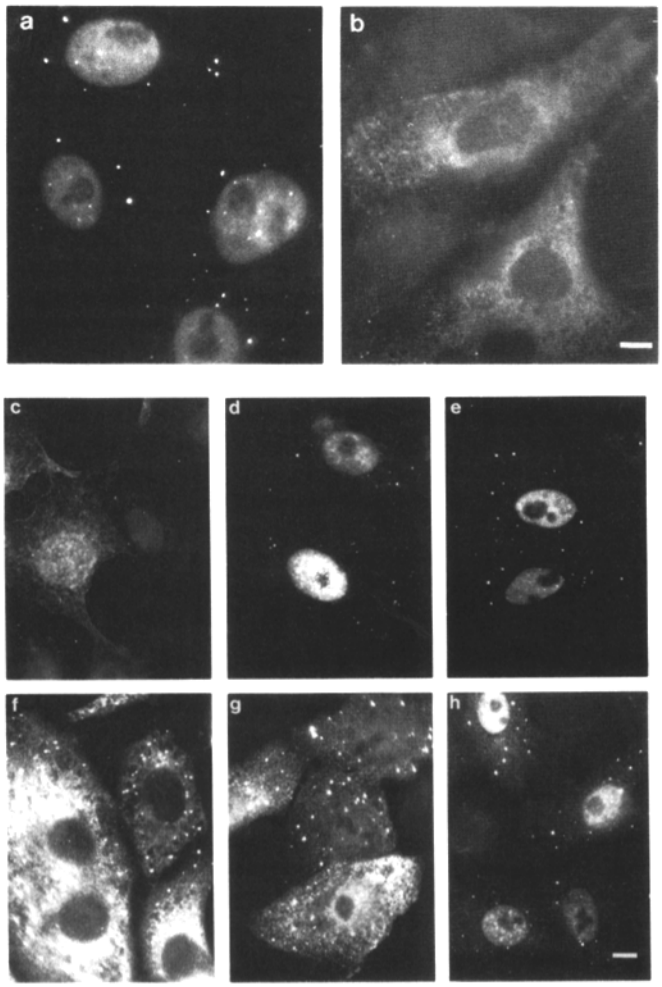

Figure 2. Nuclear transport of digoxygenin-labeled U1 snRNPs reconstituted in vitro. (*A*) Digoxygenin-labeled m_3GpppG-capped U1 RNA molecules were reconstituted in vitro as described in Materials and Methods and injected without further purification into the cytoplasm of Vero cells (1×10^5 RNA molecules/cell). Cells were incubated for 3 h at 37°C (*a*) or 4°C (*b*) and then stained with anti-digoxygenin- and FITC-conjugated anti-mouse antibody. (*B*) Comparison of the nuclear transport kinetics of U1 RNA and U1 snRNPs reconstituted in vitro. Cells were microinjected with digoxygenin-labeled m_3GpppG capped U1 RNA (*f–h*) and U1 snRNPs reconstituted in vitro (*c–e*) and incubated for 15, 30, and 60 min (*c–e*) or 30, 60, and 180 min (*f–h*) before being fixed and processed for immunofluorescence as described above. Bar, 10 μm.

Transport of U1 snRNPs Is a Mediated and Saturable Process in Somatic Cells

Having shown that U1 snRNPs reconstituted in vitro can serve as karyophiles in somatic cells, we were now able to analyze the nuclear targeting requirements for U1 snRNP particles in more detail. Initially, we asked whether the transport of in vitro reconstituted U1 snRNP is a mediated process and can therefore be inhibited at 4°C. It was only possible to carry out this experiment by using U1 snRNP particles reconstituted in vitro, since the binding of proteins to injected U1 RNA in oocytes is greatly reduced at 4°C (Fischer, U., unpublished observation). m_3G-capped U1 snRNP reconstituted in vitro was injected into the cytoplasm of Vero cells. After incubation for 3 h at 4°C, the cells were fixed and the subcellular distribution of U1 snRNP was analyzed by immunofluorescence. As shown in Fig. 2 *b*, the injected particle remained almost entirely in the cytoplasm under these conditions. This inhibition was fully reversible within 1 h after elevating the temperature to 37°C (not shown) demonstrating that the transport process of U1 snRNP in Vero cells is mediated and not due to diffusion.

The next question we asked was whether the U1 snRNP transport is a saturable process. This would give an indication for a limiting transport factor which binds to the U1 snRNP particle. For this purpose, we attempted to repress the nuclear transport of the digoxygenin-labeled U1 snRNPs by competition with nonlabeled U1 snRNPs. The latter had been purified from HeLa nuclear extracts and concentrated by MonoQ chromatography and ultracentrifugation to about 20 mg/ml; these and the labeled in vitro reconstituted U1 snRNPs were co-injected into Vero cells. The rationale behind this experiment was that if the cell's capacity to transport U1 snRNP is limited, then the transport of the labeled particles should be reduced by saturation of the transport apparatus. In the presence of a 20-fold molar excess of competitor U1 snRNP (~2 μM U1 snRNP/cell), the transport of digoxygenin-U1 snRNP reaches after 30 min an N/C value of ~1.1 (ratio of nuclear to cytoplasmic digoxygenin-labeled concentration, as determined by quantitative fluorescence measurement), that is, the concentrations in the nuclear and cytoplasmic fraction were nearly the same (Fig. 3 *A*, *c*). In

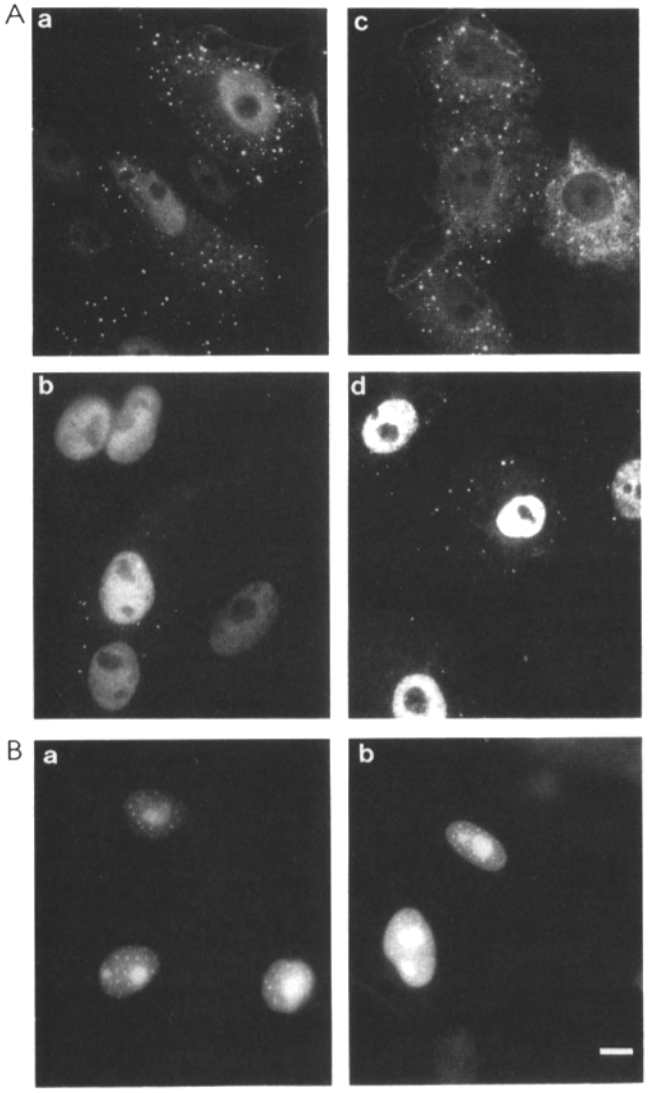

Figure 3. Nuclear transport of in vitro reconstituted U1 snRNPs is a saturable process and does not interfere with the transport of karyophilic proteins. (*A*) 1 μM solution of m_3GpppG-capped digoxygenin-labeled U1 snRNPs reconstituted in vitro was microinjected either alone (*a* and *b*) or along with 20 μM of unlabeled affinity-purified U1 snRNP particles (*c* and *d*) into the cytoplasm of Vero cells. After an incubation of 30 (*a* and *c*) or 60 (*b* and *d*) minutes at 37°C the cells were fixed and stained for immunofluorescence as described in Fig. 1. (*B*) FITC-labeled BSA conjugated with NLS-sequences (1 μM) was injected into the cytoplasm of Vero cells either alone (*a*) or along with 20 μM unlabeled affinity-purified U1 snRNP particles (*b*). Cells were incubated for 30 min at 37°C before they were analyzed by fluorescent microscopy. Bar, 10 μm.

contrast, when cells were injected with digoxygenin-U1 snRNP alone, the N/C ratio was 2.5 after the same incubation time (Fig. 3 *A*, *a*). As expected, the difference was found to level off if incubation was prolonged (Fig. 3 *A*, compare *b* and *d*) and was smaller if lower amounts of competitor U1 snRNPs were used (not shown). This clearly demonstrates that the transport of U1 snRNP is a mediated process and that U snRNP transport factors in Vero cells are present in limiting amounts.

The transport saturation observed in Fig. 3 *A* appears to be snRNP specific: a karyophilic protein such as FLUOS-labeled BSA conjugated with 8–10 NLS sequences from SV-40 T-antigen (BSA–NLS) is also transported to the nucleus in the presence of an excess of non-labeled U1 snRNP (the same amount of competitor U1 snRNP was co-injected as used for competition with the labeled U1 snRNP). Neither the kinetics nor the final extent of nuclear accumulation was affected (Fig. 3 *B*, *a* and *b*, and data not shown). The same observation was made when a karyophilic protein carrying only one NLS (P4, β-galactosidase fused with the SV-40 T-antigen NLS [Rhis and Peters, 1989]) was used (data not shown). Thus our data indicate that, in somatic cells as in *X. laevis* oocytes, distinct transport factors mediate the transport of U1 snRNP and karyophilic proteins.

The m_3G-cap Contributes to the Nuclear Uptake of U1 snRNP Even Though It Has No Essential Signaling Function in Somatic Cells

Having established that the U1 snRNP transport in somatic cells is a temperature-dependent and -mediated process, we investigated in more detail the nuclear import signal requirements of the U1 snRNP particle, in particular the role of the m_3G-cap.

In vitro reconstituted U1 snRNPs containing ApppG-capped U1 RNA were injected into the cytoplasm of Vero cells and incubated for 3 h. Surprisingly, the particle was targeted to the nucleus to an extent comparable to that of U1 snRNPs with the m_3GpppG-cap (Fig. 4 *A*, *a* and *b*). This result indicated that in contrast to the situation in oocytes, the m_3G-cap is not essential for the nuclear uptake of U1 snRNP in Vero cells. As expected, nuclear import of both m_3GpppG- and ApppG-capped U1 snRNPs was blocked if injected cells were incubated at 4°C instead of 37°C (Fig. 4 *A*, *c* and *d*). In agreement with the above findings, co-injection of a 10 mM solution of isolated m_3GpppG cap did not inhibit the transport of U1 RNA after 3 h (Fig. 4 *A*, *e*). The same amount of m_3GpppG-cap would inhibit the transport of U1 snRNP in oocytes completely (Fischer and Lührmann, 1990).

Although the m_3G-cap is not essential for nuclear import, it does enhance the transport kinetics of U1 snRNPs. In a comparison of the transport kinetics of m_3GpppG- and ApppG-capped in vitro reconstituted U1 snRNPs, ApppG-capped particles showed significantly slower transport kinetics than those bearing an m_3GpppG-cap (Fig. 4 *B*). After 15 min the m_3GpppG-capped U1 snRNPs reached an N/C ratio of approximately 1 (row *a*). The same accumulation of ApppG-capped U1 snRNP was not observed until 45–60 min after injection (row *b*). After 3 h, however, no (or only marginal) differences in the N/C ratios of the different particles were observed.

We next wished to know whether the differences in the m_3G-cap requirement for nuclear transport of U1 RNA were also observable in human 3T3 and *Xenopus* A6 cells. This would give an indication of whether the differences in the signal requirement of U1 snRNP is cell or species specific. We injected in vitro reconstituted U1 snRNPs carrying either an ApppG- or an m_3GpppG-cap into 3T3 and A6 cells and analyzed the nuclear transport as described for Vero cells. As shown in Fig. 4 *C* the U1 snRNP-particles

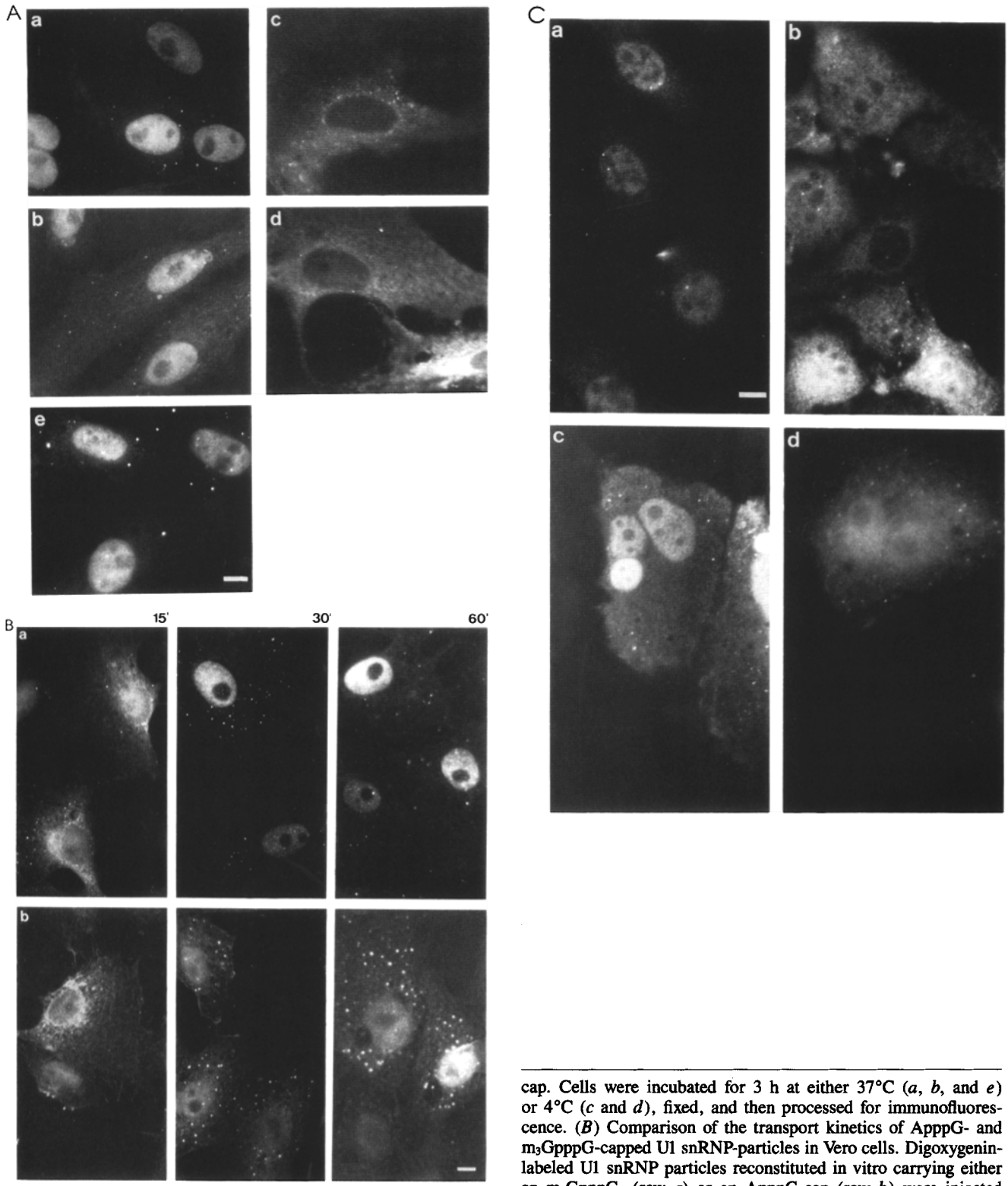

Figure 4. The m_3G-cap structure of U1 snRNP is not strictly required for nuclear targeting but accelerates the transport kinetics in Vero 3T3, and A6 cells. (*A*) ApppG-capped U1 snRNPs reconstituted in vitro are transported actively to the nucleus of Vero cells. In vitro transcribed digoxygenin-labeled U1 RNA molecules carrying either an m_3GpppG-cap (*a* and *c*) or the synthetic ApppG-cap (*b* and *d*) were reconstituted in vitro and injected into the cytoplasm of Vero cells ($\sim$1 $\times$ 10^5 RNP particles/cell). In *e* the m_3GpppG-capped U1 snRNP was coinjected with 10 mM free m_3GpppG-cap. Cells were incubated for 3 h at either 37°C (*a*, *b*, and *e*) or 4°C (*c* and *d*), fixed, and then processed for immunofluorescence. (*B*) Comparison of the transport kinetics of ApppG- and m_3GpppG-capped U1 snRNP-particles in Vero cells. Digoxygenin-labeled U1 snRNP particles reconstituted in vitro carrying either an m_3GpppG- (row *a*) or an ApppG-cap (row *b*) were injected into the cytoplasm of Vero cells and incubated at 37°C for 15, 30, and 60 min, respectively, fixed, and processed for immunofluorescence. (*C*) Nuclear transport of m_3GpppG-and ApppG-capped U1 snRNPs in mouse 3T3 and *Xenopus* A6 cells. Digoxygenin-labeled U1 snRNPs reconstituted in vitro carrying either an m_3GpppG- (*a* and *c*) or an ApppG-cap (*b* and *d*) were injected into the cytoplasm of either 3T3 cells (*a* and *b*) or A6 cells (*c* and *d*) and incubated at 37°C for 60 min. Cells were hereafter fixed and processed for immunofluorescence. Bar, 10 μm.

were targeted in 3T3 cells to the nucleus in a fashion very similar to that observed in Vero cells: both m_3GpppG-capped and ApppG-capped U1 snRNPs were targeted to the nucleus, however the ApppG-capped particle was significantly less efficiently transported (Fig. 4 *C*, *a* and *b*). A similar situation was also observed when we analyzed the nuclear transport of U1 snRNP in A6 cells. Again, both the m_3GpppG and the ApppG capped particles were targeted to the nucleus but the latter significantly slower (Fig. 4 *C*, *c* and *d*). We note that in A6 cells the kinetics of the nuclear accumulation of U1 snRNPs in general was much slower, when compared to Vero and 3T3 cells. A substantial amount of m_3GpppG-capped U1 snRNP was still detectable in the cytoplasm after 4 h, while in Vero and 3T3 cells essentially all of the injected particles accumulate in the nucleus after only 1 h (compare Fig. 4 *C*, *c* with Fig. 2 *B*). In sum, our data show that the nuclear transport of U1 snRNP in Vero, 3T3, and A3 cells appears to be less strictly dependent on the presence of the m_3G-cap than in oocytes. However, the m_3G-cap does accelerate the transport kinetics of U1 snRNP indicating that it has retained a signaling role for nuclear targeting of U1 snRNPs in somatic cells. Moreover, our data strongly suggest that cell specific rather than species specific differences account for the differential m_3G-cap requirement in nuclear import of U1 snRNPs.

One possible explanation for the differential requirement for the m_3G-cap in U1 snRNP transport in somatic cells versus *X. laevis* oocytes could be due, at least in part, to the differential contribution of U1 specific proteins to nuclear transport in the two cell systems. Given that some of the U1 specific proteins also contain NLSs (Kambach and Mattaj, 1991; Jantsch and Gall, 1991), one could envisage the possibility that in somatic cells these signals might override the m_3G-cap signal and therefore obviate the need for the cap in U1 snRNP transport. To address this question we analyzed the transport of an artificial RNA that contained the 3′-terminal 42 nucleotides of U1 RNA (encompassing the Sm site and stem/loop E) and an artificially designed 5′ terminal stem/loop structure 22–nucleotides long (Fischer et al., 1993). This RNA designated as SmII RNA, can bind only the common but not the specific U snRNP proteins and therefore enabled us to analyze the contribution of the Sm core domain in nuclear targeting, while any contribution of the U1 specific proteins can be excluded.

We have previously observed that injection of naked SmII RNA in oocytes yielded transport deficient SmII RNP particles in the ooplasm. Moreover, immunoprecipitation experiments revealed that the in vivo assembly of SmII RNP in oocytes was less efficient as compared to U1 RNA (Fischer et al., 1993). It was therefore not surprising that no nuclear transport was observed either when naked SmII RNA was injected into Vero cells. In fact, cells injected with naked digoxygenin-labeled SmII RNA could not be stained by immunofluorescence, suggesting that this RNA is rapidly degraded in situ (not shown). In contrast, in vitro prepared SmII snRNPs proved to be more stable and could be detected by immunofluorescence, consistent with our observation that in vitro reconstituted SmII RNPs are also efficiently transported in *Xenopus* oocytes (Fischer et al., 1993). In the following transport experiments, we therefore used exclusively the SmII snRNP particle prepared in vitro. As shown in Fig. 5 *A* (*a*) the in vitro prepared m_3GPpppG-capped SmII

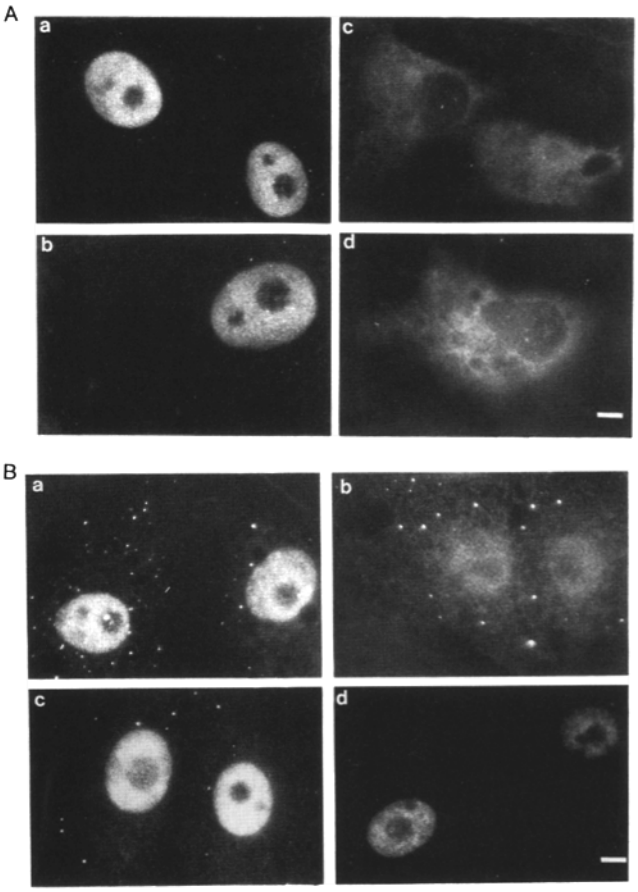

Figure 5. A minimal SmII RNA which binds exclusively the common proteins is targeted to the nucleus after reconstitution in vitro. (*A*) In vitro transcribed digoxygenin-labeled SmII RNA carrying an m_3GpppG-cap was injected after reconstitution in vitro into the cytoplasm of Vero cells (*a* and *c*). Cells shown in *b* and *d* were injected with in vitro reconstituted ApppG-capped SmII RNP. Cells were incubated for 1 h at 37°C (*a* and *b*) or 4°C (*c* and *d*) before they were fixed and processed for immunofluorescence. (*B*) Saturation of SmII snRNP transport by co-injection of excess isolated U1 snRNP. A 1-μM solution of m_3G-capped digoxygenin-labeled SmII snRNP reconstituted in vitro was injected either alone (*a* and *c*) or along with 20 μM of unlabeled U1 snRNP into the cytoplasm of Vero cells (*b* and *d*). After 30 (*a* and *b*) and 120 min (*c* and *d*) cells were fixed and analyzed by immunofluorescence. Bar, 10 μm.

snRNP was as efficiently transported to the nucleus as in vitro reconstituted U1 snRNPs after 1 h. This transport also occurred independently of the cap structure of the SmII RNA as ApppG- and m_3GpppG-capped SmII snRNPs were both transported to the nucleus to a comparable extent within 1 h (Fig. 5 *A*, *b*). To exclude any uptake due to passive diffusion, we incubated Vero cells injected with SmII snRNP at 4°C and analyzed the transport 1 h later. As expected, the transport was severely inhibited excluding diffusion of the SmII snRNP into the nucleus (Fig. 5 *A*, *c* and *d*). In addition the transport of SmII snRNP was also inhibited to a significant extent by co-injection of an excess of unlabeled U1 snRNP (Fig. 5 *B*, compare *a* and *c* with *b* and *d*). These results resembled strongly the saturation of digoxygenin-labeled U1 snRNP transport shown in Fig. 3 in that the same amount of competitor U1 snRNP was needed to obtain a comparable

inhibition of transport. As U1 snRNP can compete with SmII snRNP in an import reaction it is likely that both the U1 snRNP and the SmII snRNP bind the same limiting transport factor(s) and therefore use the same pathway to migrate into the nucleus. Moreover these results rule out any contribution of the U1 specific proteins of the U1 snRNP particle to nuclear targeting. Taken together, our data suggest that the Sm core domain is not only an essential signalling structure but under certain conditions, is also sufficient to bring about nuclear transport of U1 snRNP particle in Vero cells.

Discussion

In this report we have used microinjection of digoxygenin-labeled U1 RNA and snRNP and derivatives thereof to study the structural requirements of snRNA transport in somatic cells.

Several lines of experimental evidence indicate that the transport of digoxygenin-labeled snRNAs was specific and not due to possible diffusion of breakdown products and/or to nonspecific association with cellular structures: (*a*) When free U1 RNA molecules were injected into the cytoplasm they accumulated in the nucleoplasm only excluding the nucleoli. Moreover transport was observed only at 37°C but not at 4°C. These facts do not agree with the possibility that breakdown products of U1 RNA have accumulated in the nucleus in an unspecific way. (*b*) Transport of free U1 RNA is dependent on the assembly of Sm proteins as shown by the failure of U1ΔD to be transported and the finding that anti-Sm antibody Y12 strongly interferes with nuclear transport. Again this would not be expected if only fragments of degraded RNAs diffuse to the nucleus. (*c*) When free SmII RNA was injected this RNA was apparently not assembled in situ into a stable SmII complex and therefore degraded in the cell. Remarkably under these conditions no fluorescent label was detectable after 1–3-h incubation, neither in the cytoplasm nor in the nucleus. This also shows that if digoxygenin-labeled RNAs are degraded the digoxygenin-labeled nucleotides apparently are not efficiently incorporated in situ into newly transported endogenous RNA molecules. Frequently we observed that a small fraction of the injected digoxygenin-labeled RNA remained in the cytoplasm in separate dots. It is currently not clear whether this is due to either aggregation of the digoxygenin-labeled RNA with other cellular structures or precipitation of a fraction of the injected material in the cytoplasm.

A major breakthrough in our studies was the possibility to obtain in vitro reconstituted digoxygenin-labeled snRNPs at concentrations allowing microinjection into somatic cells. This enabled us to study the structural requirements for the transport of U1 snRNPs independently of the in situ assembly of snRNPs. We have good reasons to believe that the in vitro reconstituted snRNPs are transported in Vero cells as such and do not undergo a disassembly/reassembly process. First, the kinetics of nuclear transport of reconstituted snRNPs are faster as compared with injected free snRNA. Second, only the reconstituted SmII snRNP was stable in the cell and targeted actively to the nucleus whereas the free RNA was degraded (see above).

As in oocytes, the transport of U1 snRNP and SmII snRNP in somatic cells was strongly temperature dependent and saturable (Figs. 2 and 3) indicating an active and mediated process.

As shown in oocytes, U snRNPs, and karyophilic proteins use different kinetic pathways to reach the nucleus. This conclusion could be drawn by the observation that upon saturation of the protein transport the U snRNP transport could proceed and vice versa (Fischer et al., 1993; Michaud and Goldfarb, 1991, 1992). As shown in this report the same holds true also for Vero cells: when the U1 snRNP transport was saturated by injection of an excess of purified U1 snRNP the protein transport was not affected at all. This observation fits nicely with the recent finding that the expression of a frameshift mutant of the large T-antigen of SV-40 in Vero cells inhibits the transport of heterologous karyophilic proteins but not of U1 RNA (van Zee et al., 1993). In this case strong evidence has been presented that the T-antigen mutant titrates out a limiting protein specific transport factor while the U snRNP-specific transport pathway is unaffected. Furthermore, transport competition studies carried out in vitro along the same lines as described in this work are fully consistent with the above stated conclusions (Marshallsay and Lührmann, 1984).

In *X. laevis* oocytes the spliceosomal snRNAs have a differential m_3G-cap requirement for nuclear transport: whereas U1 and U2 transport is strictly dependent on the 5′-terminal m_3G-cap, U4 and U5 snRNAs can enter the nucleus also with an unphysiological ApppG-cap at their 5′-ends (Fischer et al., 1991). One of the most surprising findings in this work was that the m_3G-cap of U1 snRNP is not an essential signaling component in somatic cells. It appears that this is also true for the other spliceosomal snRNPs since U5 was also transported m_3G-cap independently to the nucleus in Vero cells (not shown). However, the m_3G-cap still has retained a signaling role for the nuclear targeting of U1 snRNP, since ApppG-capped U1 snRNPs showed significantly slower transport kinetics than m_3GpppG-capped one. Since this feature was observed in somatic cells from different species (*Xenopus* A6, mouse 3T3 and monkey Vero cells) we propose that it may apply more generally for somatic cells and is not species specific.

While the m_3G-cap is not strictly required, the Sm core domain in contrast might even be sufficient for nuclear targeting of U1 snRNP in somatic cells. This is underscored by the finding that: (*a*) U1 RNA but not U1ΔD is targeted to the nucleus; (*b*) the SmII RNP reconstituted in vitro migrates to the nucleus independently whether the RNA contained an ApppG- or an m_3GpppG-cap; and (*c*) the transport of SmII RNP was inhibited upon coinjection of saturating amounts of U1 snRNP. The Sm core domain facilitates therefore the nuclear transport of U1 snRNP by interaction with a limiting factor.

Why does the U1 snRNP require the m_3G-cap as an essential part of the NLS in oocytes but not in Vero, A6, and 3T3 cells? Structural variations of components of the transport apparatus in the different cell types is one possible explanation for these observations. Similarly, differences in the cellular factors in oocytes and somatic cells could also be the reason for the differential m_3G-cap requirement in nuclear transport of U1 snRNP. Because the Sm core domain is essential for nuclear targeting of U1 snRNP not only in somatic cells but also in the oocyte, it appears likely that differences in the recognition of this domain by a transport factor account for the m_3G-cap dependence. According to this model a Sm core domain recognizing transport factor which differs from its oocyte equivalent is present in A6 cells (and

probably in somatic cells in general). This factor is predicted to possess a higher "transport activity" which makes the cap less important. The higher "activity" of this factor could result from differences in posttranslational modification or in genetic variation. The oocyte receptor equivalent might have a lower nuclear targeting activity and makes the assistance of the m_3G-cap essential.

One prediction of the above mentioned model is that the m_3G-cap requirement for nuclear import changes during development of the oocyte to somatic cells. It is currently not yet clear at which stage in the development the switch in the signal requirement occurs nor it is known whether this process affects events in development. Interestingly, a comparison to the nuclear transport of karyophilic proteins reveals that several cases of cell type- and species-specific differences in the usage of NLS exist (Slaviak et al., 1989; Standiford and Richter, 1992; Fischer-Fantuzzi and Vesco, 1988). For example a developmentally regulated NLS was recently discovered in the E1A protein of adenovirus 5 (Standiford and Richter, 1992). This NLS functions in *Xenopus* oocytes and in cells of the embryo up to the early neurular stage but not later in development or in A6 cells. Therefore, the same situation we have observed for the transport of U1 snRNPs in different cell types may apply also for some karyophilic proteins.

The NLS of U1 snRNP, although structurally completely different probably resembles in its modular character, the bipartite NLS of karyophilic proteins (Dingwall and Laskey, 1991). The downstream cluster of protein NLS might have its equivalent in the Sm core domain and is likely to be responsible for efficient binding to the transport factors. The m_3G-cap in contrast (similar to the upstream cluster in protein NLS) might have rather a degenerative function and might be dispensable when cellular conditions or the snRNP structure allows this.

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